Nitrate addition has minimal short-term impacts on Greenland ice sheet supraglacial prokaryotes

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Title: **Nitrate addition has minimal short-term impacts on Greenland ice sheet supraglacial prokaryotes.**

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Originality-Significance statement:

Tropospheric concentrations of nitrate have increased since industrial times, and this trend is set to continue as society's demand on technology, agriculture and transport intensify. Once deposited within terrestrial ecosystems, nitrate may fertilize and consequentially alter the structure, composition and biogeochemical functioning of biotic communities. The surface of the Greenland ice sheet is colonized by active microbiota that darken surface ice and enhance melt. Alterations to the biomass or to the composition of these communities may consequently influence their impact on surface ice melt. This study is the first to report *in situ* fertilization experiments on the Greenland ice sheet to consider the affect of elevated nitrate concentrations on the abundance and composition of dominant surface ice microbiota. We find that the estimated abundance and community composition of dominant prokaryotes residing within surface ice holes was minimally affected during six weeks of incubation, and we therefore suggest that these communities would be unaffected by an exclusive rise in nitrate depositions.

Summary (180 words):

Tropospheric nitrate levels are predicted to increase throughout the 21st century, with potential effects on terrestrial ecosystems, including the Greenland ice sheet (GrIS). This study considers the impacts of elevated nitrate concentrations on the abundance and composition of dominant bulk and active prokaryotic communities sampled from *in situ* nitrate fertilization plots on the GrIS surface. Nitrate concentrations were successfully elevated within sediment-filled meltwater pools, known as cryoconite holes; however, nitrate additions applied to surface ice did not persist. Estimated bulk and active cryoconite community cell abundance was unaltered by nitrate additions when compared to control holes using a quantitative PCR approach, and nitrate was found to have minimal affects on the dominant 16S rRNA gene-based community composition. Together, these results indicate that sampled cryoconite communities were not nitrate limited at the time of sampling. Instead, temporal changes in biomass and community composition were more pronounced. As these *in situ* incubations were short (6 weeks), and

the community composition across GrIS surface ice is highly variable, we suggest that further efforts should be considered to investigate the potential long-term impacts of increased nitrate across the GrIS.

Introduction:

Atmospheric deposition of anthropogenic nitrate has increased since the 1850's as a result of heightened fossil fuel burning and fertilizer usage (Galloway *et al.*, 2008; Felix and Elliott, 2013). Nitrate depositions that occur over the Greenland ice sheet (GrIS), whether dissolved within precipitation or adsorbed to dust particles, become encapsulated within accumulating snow, and can be used as a historic record of the changing global nitrogen cycle (Hastings *et al.*, 2009; Felix and Elliott, 2013; Geng *et al.*, 2014). Tropospheric nitrate levels have been predicted to rise 181% by 2100 (Liao *et al.*, 2006), which may have consequential effects on downwind recipient ecosystems (Vitousek *et al.*, 1997; DeForest *et al.*, 2004), including the surface of the GrIS. The GrIS surface harbours spatially variable, active microbial communities (Edwards *et al.*, 2014; Chandler *et al.*, 2015; Stibal *et al.*, 2015; Cameron *et al.*, 2016), which are involved in, or have shown the potential for carbon and nutrient cycling (Cameron *et al.*, 2012a; Stibal *et al.*, 2012; Telling *et al.*, 2012). These communities contribute towards surface ice darkening and so enhance melt (Yallop *et al.*, 2012; Musilova *et al.*, 2016). Furthermore, supraglacial microbiota can collate into 'cryoconite' aggregates within shallow, water filled holes, where increased cryoconite mass within each hole leads to horizontal expansion of the ice structure through localized melt (Cook *et al.*, 2010; Cook *et al.*, 2015).

In order to examine the effect of increased nitrate concentrations on the dominant prokaryotic abundance and community structure of the GrIS, we performed short-term (6 week) *in situ* nitrate fertilization experiments within cryoconite holes and on surface ice in the southwestern margin of the GrIS. We simulated increased nitrate concentrations in line with expected concentrations for 2100 (low nitrate addition), as well as a ten-fold increase on current day levels (high nitrate addition; experimental

procedures can be found within the Supporting Information). We hypothesized that increasing nitrate concentration will promote biomass production and influence the community structure.

Results and discussion:

Nitrate concentrations within cryoconite holes were successfully amended. Prior to nitrate additions, the mean concentration of nitrate from all nine cryoconite holes was 0.13 ± 0.04 mg L^{-1}. After nitrate was added, control holes maintained a mean nitrate concentration of 0.12 ± 0.05 mg L^{-1}, while holes with low and high nitrate additions became more nitrate concentrated (mean concentration; 8.94 ± 12.10 mg L^{-1}, and 45.27 ± 72.96 mg L^{-1} respectively; Fig. 1). Due to high variability, the nitrate concentrations were not significantly different between low and high addition holes (two tailed paired t-test; $t = 2.18$; $p = 0.08$); however, nitrate concentrations were significantly different between control holes and holes that had either low or high nitrate additions (two tailed t-test; $t = 3.64$; $p = 0.01$). The cryoconite experimental conditions are therefore considered as either control or nitrate treatments herein. Nitrate treatments administered to the surface ice were not retained for more than 24 hours. Prior to nitrate additions, the mean concentration of nitrate from the nine surface ice plots was 0.06 ± 0.02 mg L^{-1}, which was within the same range as ice sampled along a nearby transect (Telling *et al.*, 2012). The mean nitrate concentration of all surface ice samples after treatment addition was 0.11 ± 0.07 mg L^{-1}, and there was no statistical difference in the nitrate concentrations measured between different treatment groups (one-way paired ANOVA; $R^2 = 0.11$, $p < 0.01$), therefore further analysis of these samples was not considered.

Short-term elevated concentrations of nitrate had no significant affects on the estimated abundance of 16S rRNA genes from cryoconite biota. Cryoconite bulk cell abundance, estimated from DNA extracts, did not significantly vary between control and nitrate treatments (two tailed paired t-test; $t = 0.91$; $p = 0.40$; Fig. 2a). Under both treatments the total estimated abundance increased over the course of the sampling with the mean estimated abundance for all communities changing from $1.77 \times 10^{10} \pm 0.51 \times 10^{10}$ cell g^{-1} on day of year (DOY) 180 to $5.27 \times 10^{10} \pm 1.38 \times 10^{10}$ cell g^{-1} on DOY 222. The number of active cells,

estimated from cDNA extracts, similarly did not significantly vary between experimental conditions (two tailed paired t-test; $t = 0.84$; $p = 0.44$; Fig. 2b). Estimated bulk cell abundance and active cell abundance correlated tightly (Pearson's correlation; $r = 0.86$; $p = 0.01$), with both peaking on DOY 208 (mean estimated abundance; $5.75 \times 10^{10} \pm 1.89 \times 10^{10}$ cell g^{-1} and $5.87 \times 10^{10} \pm 1.85 \times 10^{10}$ cell g^{-1} respectively). We find that estimated cell abundance calculated from bulk and active communities was in line with cryoconite sampled ~12 km north (Stibal *et al.*, 2015), and that bulk communities were an order of magnitude more abundant than cryoconite sampled from Svalbard (Zarsky *et al.*, 2013), and were several orders of magnitude more abundant than cryoconite sampled from the southwestern margin of the GrIS when epifluorescence microscopy was used for analysis (Musilova *et al.*, 2015). We note, however, that no steps were taken to reduce or quantify the amplification of extracellular DNA (e.g. Nielsen *et al.*, 2007; Kim *et al.*, 2016); therefore our values may overestimate abundance. The lack of response to nitrate addition indicates that the majority of the communities were either not nitrate limited, or they are co-limited by nitrate and another nutrient. The uptake and storage of NH_4^+, originating from atmospheric depositions, from melting snowpack or from organic mineralization, into cryoconite, should be considered as an addition source of bioavailable nitrogen for these sampled systems, especially given their inland location within a region where nitrogen fixation activities have not previously been detected (Wynn *et al.*, 2007; Hodson *et al.*, 2010; Telling *et al.*, 2011; Telling *et al.*, 2012). Supraglacial phosphorus and carbon limitations have been identified using cryoconite from Svalbard (Mindl *et al.*, 2007; Säwström *et al.*, 2007; Stibal and Tranter, 2007; Stibal *et al.*, 2009). We therefore suggest that future elevations of nitrate aerosols (Liao *et al.*, 2006), in the absences of additional phosphorus and carbon sources, may have little effect on the biomass of similar GrIS cryoconite systems.

Nitrate addition was found to have minimal effects on dominant prokaryotic community composition in cryoconite. DNA and cDNA 16S rRNA gene amplicon libraries of cryoconite samples taken after three weeks of treatment were moderately similar between control and nitrate conditions when sample date was accounted for in two-way ANOSIM analyses (DNA: *Global R* = 0.31; $p < 0.01$, cDNA: *Global R* = 0.41; p

< 0.01). One-way ANOSIM analysis of amplicon libraries grouped by treatment identified high similarity between control and nitrate conditions (DNA; $R = 0.13$; $p = 0.01$, cDNA; $R = 0.17$; $p < 0.01$), whereas one-way ANOSIM analysis of amplicon libraries grouped by sample date identified moderate similarity between control and nitrate additions (DNA; $R = 0.30$; $p = 0.01$, cDNA; $R = 0.26$; $p < 0.01$). As this investigation targeted changes in dominant community members, it is likely that the impact of increased nitrate on denitrifiers will not have been detected; considering that copy numbers from potential denitrifiers represented <1%, judged from the 16S rRNA gene copy numbers amplified from glacial forefield soils (Kandeler *et al.*, 2006). Nevertheless, the potential of cryoconite microbial communities to utilize nitrate has been reported previously as a result of *nirS* and *nosZ* genes analysis (Cameron et al., 2012a), as well as through biogeochemical investigations (Telling et al., 2012), and in this current study we consider whether there are downstream implications to the community as a whole. Principal component analysis (PCA) identified that 80% of the variance in the DNA amplicon libraries could be explained within the first 4 axes when samples taken prior to treatment additions were excluded. To identify the most significant factors influencing microbial community structure, a redundancy analysis (RDA) was performed using sampling date (as day of year 2014), nitrate treatment, and chloride, nitrate and sulphate concentrations (Supporting Information Table 1) as the explanatory variables. Together these variables were found to account for 32.1% of variance (*pseudoF* = 4.1, $p = 0.001$). Interactive forward selection identified that sampling date was the most significant factor influencing community variability, explaining 18.1% of variance (*pseudoF* = 10.4, $p = 0.001$), followed by the nitrate treatment, which explained 9.8% of variance (*pseudoF* = 6.3, $p = 0.001$). For the cDNA amplicon communities, 89% of variance in community structure was explained within the first 4 axes of a PCA. RDA identified that 38.6% of variance between communities was explained by the used explanatory factors, with sampling date contributing towards the greatest influence (28.0% of variance explained, *pseudoF* = 17.9, $p = 0.001$), followed by the nitrate treatment (5.4% of variance explained, *pseudoF* = 3.7, $p = 0.02$). Temporal changes in the estimated absolute abundance of predominant bulk and active community members are shown in Fig. 3a, and the homogeny between the estimated absolute abundance of predominant community

members under differing nitrate treatments is shown in Fig. 3b. The higher percentage of variance that was explained by sampling date in the analysis of active communities, compared to the analysis of bulk communities, is suggestive that active communities are more temporally variable. Diversity analysis and PCA of bulk prokaryotic cryoconite communities sampled from the southwestern margins of the GrIS by Musilova *et al*., (2015) have also shown these systems to vary temporally. The disparity between total variation and explained constrained variation in both bulk and active amplicon libraries is suggestive that additional factors, such as hole age and dimension, temporal variability and surface ice hydrology, that have not been targeted within this study, may be important for structuring these cryoconite communities.

16S rRNA gene amplicons from dominant bulk and active prokaryotic cryoconite communities were identified as being highly distinct from each other in two-way ANOSIM analyses that accounted for either experimental treatment or sampling date (*Global R* = 0.97; $p < 0.01$, *Global R* = 0.98; $p < 0.01$, respectively; Fig. 3). Amplicon libraries from DNA extracts were calculated to be more diverse than those from cDNA extracts using CatchAll analysis (mean diversity of amplicon libraries from DNA extracts; 97.01 ± 18.86, mean diversity of amplicon libraries from cDNA extracts; 71.44 ± 20.82, two tailed t-test; $t = 7.09$; $p < 0.01$). Libraries generated from DNA extracts were predominated by amplicons related to Pseudanabaenaceae of the genus *Leptolyngbya* (39.92 ± 6.13%; $1.52 \times 10^{10} \pm 0.95 \times 10^{10}$ cells g^{-1}; likely *Phormidesmis priestleyi*; Chrismas *et al*., 2015; Gokul *et al*., 2016; Uetake *et al*., 2016), Thermogemmatisporaceae (27.27 ± 6.31%; $1.09 \times 10^{10} \pm 0.77 \times 10^{10}$ cells g^{-1}), Sphingomonadaceae of the genera *Novosphingobium* and *Sphingomonas* (10.97 ± 4.72%; $2.43 \times 10^{9} \pm 1.42 \times 10^{9}$ cells g^{-1}) and Nostocaceae (9.34 ± 7.28%; $2.32 \times 10^{9} \pm 3.03 \times 10^{9}$ cells g^{-1}), while libraries generated from cDNA extracts were predominated by *Leptolyngbya* (79.69 ± 10.22%; $2.95 \times 10^{10} \pm 1.87 \times 10^{10}$ cells g^{-1}; Fig. 3). Although members of the Nostocaceae and *Leptolyngbya boryana*, a close relative of *P. priestleyi*, are capable of nitrogen fixation (Stewart and Lex, 1970), Telling *et al*. (2012) reported that nitrogenase activity was not detected beyond ~7 km inland from the margins of the GrIS, when a transect that

crossed ~7 km north of the current sample site was performed in early August 2010. Furthermore, no evidence for nitrogen fixation genes was found within the genome of *P. priestleyi* isolated from cryoconite sampled in close proximity to the current study (Chrismas *et al*., 2016). We therefore presume that cyanobacterial cells within bulk and active communities were not fixing nitrogen, and that the measured nitrate originated from ice, snow and rain (Telling *et al.*, 2011; Telling *et al.*, 2012). Cyanobacteria, Chloroflexi and Bacteroidetes dominated GrIS cryoconite communities have been reported previously (Stibal *et al.*, 2015; Cameron *et al.*, 2016). However, neither Cyanobacteria nor Chloroflexi dominated GrIS cryoconite communities were sampled from Leverett Glacier, 36 km away (Musilova *et al.*, 2015), and other GrIS sampled communities have been solely Cyanobacteria dominated (Cameron *et al.*, 2012b; Uetake *et al.*, 2016), strengthening previous arguments of the spatial variability of cryoconite communities across the GrIS (Cameron *et al.*, 2016).

In this study we adopted an *in situ* approach to minimize experimental artefacts prior to sampling. Glacial ice surfaces are dynamic and topographically and hydrologically changeable environments (Fountain *et al.*, 2004; Tranter *et al.*, 2004; Bagshaw *et al.*, 2007; Cook *et al.*, 2015). Consequently, cryoconite holes and ice surfaces underwent ablation and were flushed with melt water throughout the study period. While efforts were made to maintain the desired levels of nitrate by fertilizing study sites every 24 hours, future designs of *in situ* experiments should consider alternative approaches in order to better achieve desired nutrient levels. In this study, elevated levels of nitrate were not maintained on the surface ice, however, considering the physical differences of these niches in comparison to cryoconite holes, such as the reduced availability of liquid water, and their contrasting community compositions (Yallop *et al.*, 2012; Cameron *et al.*, 2016), surface ice communities may respond differently to elevated nitrate concentrations, and this may be spatially variable (Cameron *et al.*, 2016). In particular, algae thrive upon surface ice and snow (Yallop *et al.*, 2012; Lutz *et al.*, 2014; Lutz *et al.*, 2016), and these communities, like algal communities of oceans, river and lakes, may respond significantly to increased nitrate availability, which may have notable effects on the darkening and therefore the melting of ice and snow surfaces

(Yallop *et al.*, 2012; Lutz *et al.*, 2014; Lutz *et al.*, 2016). While the effects of nitrate addition could only be monitored within a relatively short time-scale in this current study, it is interesting to note that previous reports have suggested that increased depositions of anthropogenic nitrate on glacier surfaces since preindustrial times may have reduced the necessity of surface ice microbial communities to fix nitrogen (Telling *et al.*, 2011). Further to this, snow and rain are the main nitrate contributors to supraglacial environments (Hodson *et al.*, 2005), and as precipitation within the Arctic is predicted to increase throughout the 21st century (Kattsov *et al.*, 2007; Schuenemann and Cassano, 2010), this too may amplify available nitrate sources on the ice. As bare ice areas around the margins of the GrIS continue to expand in response to changes in climate (Box *et al.*, 2012), communities close to the margin will have to endure longer periods without nitrate enrichment from snowmelt (Telling *et al.*, 2012; Telling *et al.*, 2014), whereas newly exposed inland ice surfaces will experience enrichment from both snowmelt and from the melting of ice that post-dates mid 19th century anthropogenic nitrate increases (Galloway *et al.*, 2008; Felix and Elliott, 2013). Elevated surface ice temperatures and increased meltwater availability are similarly key factors that may influence surface ice ecology in the future (Säwström *et al.*, 2007; Hall *et al.*, 2013).

In summary we find that short-term incubations of cryoconite in elevated concentrations of nitrate had no significant affect on the estimated abundance, and little effect on the composition of dominant bulk and active prokaryotes, which is suggestive that these systems are not nitrate limited, or that they are co-limited by another nutrient.

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Figures:

Fig. 1: Mean nitrate concentrations of sampled cryoconite holes. White denotes control treatments (no nitrate added), grey denotes low treatments (0.8 M nitrate added every 24 hours), and black denotes high treatments (4.6 M nitrate added every 24 hours). Error bars show standard deviations. The limit of detection is shown by the dotted line (0.05 mg L^{-1}). Nitrate concentrations are shown on a $\log_{10}$ scale. n=3, except for the final two control measurements where n=2.

Fig. 2: Estimated cell abundance of (a) bulk communities and (b) active communities throughout the experimental period. Gene copy numbers were quantified using qPCR analysis of 16S rRNA genes and cell abundance was estimated using gene copy numbers of parallel 16S rRNA gene diversity profiles. Circles denote individual values and bars denote mean estimated cell abundance per time point per treatment. White circles and dashed lines denote control treatments (no nitrate added), grey circle and bars denote low treatments (0.8 M nitrate added every 24 hours), and black circles and solid bars denote high treatments (4.6 M nitrate added every 24 hours).

Fig. 3: Absolute estimated cell abundance of OTU grouped by family-level or phylum-level ([p]) taxonomy. (a) Estimated bulk community abundance, grouped by taxa and with sets of bars showing mean estimated abundance by day of sampling ordered left to right by day of year (2014); 180, 187, 194, 201, 208, 214, 222, regardless of nitrate treatment. Inset graph shows estimated active community abundance. (b) Estimated bulk community abundance, grouped by taxa and with sets of bars showing mean estimated abundance after three weeks of nitrate treatment, ordered left to right; control (no nitrate added), low (0.8 M nitrate added every 24 hours), high (4.6 M nitrate added every 24 hours). Inset

graph shows estimated active community abundance. Numbers in brackets relate to the number of OTU included in each taxa group, error bars show standard deviation.

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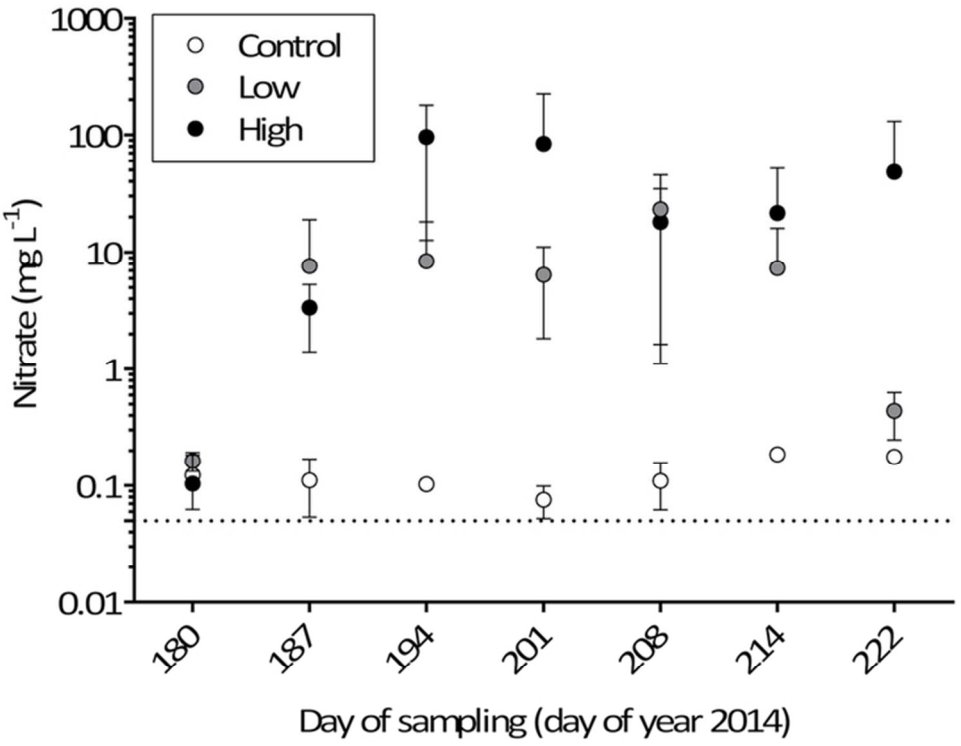

Fig. 1: Mean nitrate concentrations of sampled cryoconite holes. White denotes control treatments (no nitrate added), grey denotes low treatments (0.8 M nitrate added every 24 hours), and black denotes high treatments (4.6 M nitrate added every 24 hours). Error bars show standard deviations. The limit of detection is shown by the dotted line (0.05 mg L^{-1}). Nitrate concentrations are shown on a $\log^{10}$ scale. n=3, except for the final two control measurements where n=2.

Fig. 1
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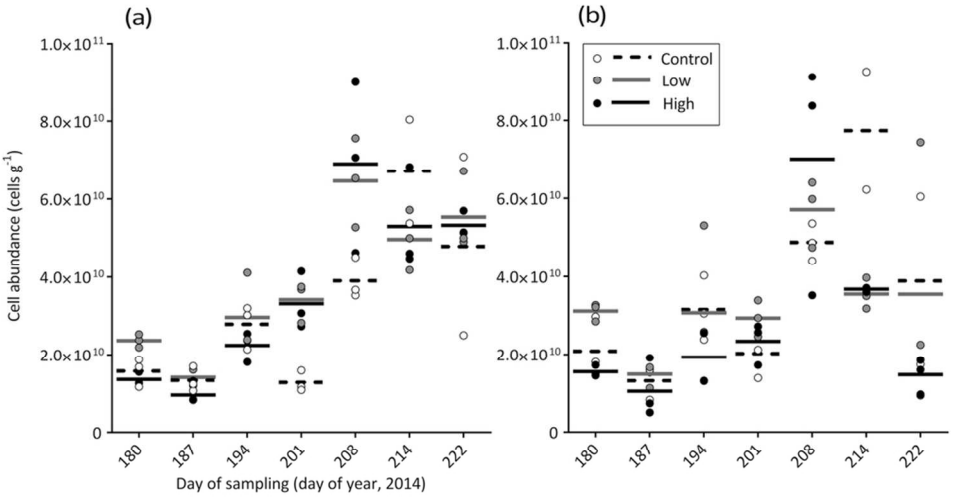

Fig. 2: Estimated cell abundance of (a) bulk communities and (b) active communities throughout the experimental period. Gene copy numbers were quantified using qPCR analysis of 16S rRNA genes and cell abundance was estimated using gene copy numbers of parallel 16S rRNA gene diversity profiles. Circles denote individual values and bars denote mean estimated cell abundance per time point per treatment. White circles and dashed lines denote control treatments (no nitrate added), grey circle and bars denote low treatments (0.8 M nitrate added every 24 hours), and black circles and solid bars denote high treatments (4.6 M nitrate added every 24 hours).

Fig. 2
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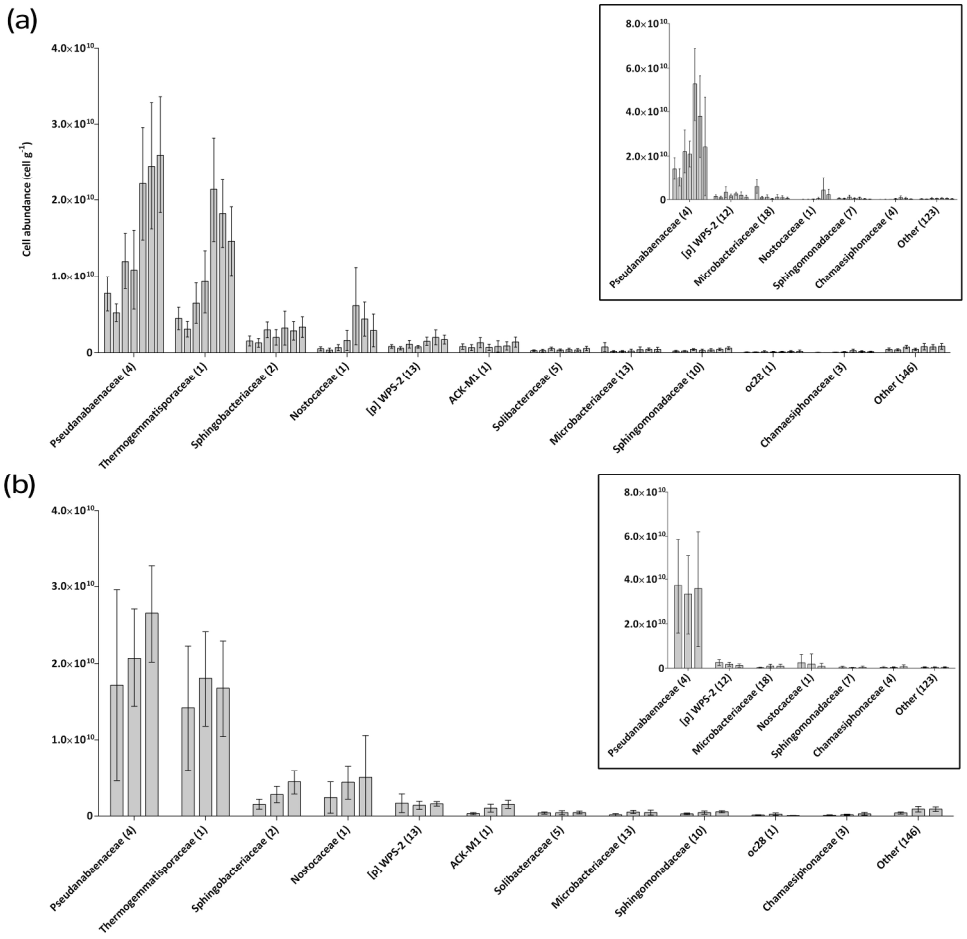

Fig. 3: Absolute estimated cell abundance of OTU grouped by family-level or phylum-level ([p]) taxonomy. (a) Estimated bulk community abundance, grouped by taxa and with sets of bars showing mean estimated abundance by day of sampling ordered left to right by day of year (2014); 180, 187, 194, 201, 208, 214, 222, regardless of nitrate treatment. Inset graph shows estimated active community abundance. (b) Estimated bulk community abundance, grouped by taxa and with sets of bars showing mean estimated abundance after three weeks of nitrate treatment, ordered left to right; control (no nitrate added), low (0.8 M nitrate added every 24 hours), high (4.6 M nitrate added every 24 hours). Inset graph shows estimated active community abundance. Numbers in brackets relate to the number of OTU included in each taxa group, error bars show standard deviation.

Fig. 3
129x128mm (600 x 600 DPI)